NON-ANAPHYLACTIC FORMS OF ALLERGENS AND THEIR USE.

Technical field and background

The invention concerns non-anaphylactic forms of protein allergens and the use of the forms for hyposensitization and for determining antibodies (IgA, IgD, IgE, IgG, IgM) directed against the allergen, for instance in the context of diagnosing in vitro type I allergy (IgE mediated allergy). The invention also concerns a method for hyposensitization of a mammalian individual, typically a human individual, suffering from type I allergy against a protein allergen.

The invention primarily concerns treating and diagnosing humans.

By a protein allergen is meant any protein/polypeptide causing a type I mediated allergic reaction. Thus the term encompasses any naturally occuring protein allergen including the smallest fragments thereof that will cause a type I allergic reaction in a mammal, most importantly humans.

In April 1997, the present inventors have published an article dealing with non-anaphylactic fragments of the Bet v 1 allergen. See Vrtala et al., "Conversion of the major birch pollen allergen, Bet v 1, into two non-anaphylactic T cell epitope containing fragments", J. Clin. Invest. 99(7) April 1997) 1673-1681.

Type I allergy represents a major health problem in industrialised countries where more than 20 % of the population suffer from Type I allergic reactions (allergic rhinitis, conjunctivitis, allergic asthma and anaphylactic shock) (Kaplan (ed) Allergy. Churchill Livingstone, New York (1985)). Environmental proteins from pollen, mites and animal dander belong to the major components which induce

release of biological mediators (e.g. histamine) by crosslinking effector cell (mast cell, basophil) bound specific IgE antibodies. The production of specific IgE from B-cells is stimulated by allergen specific T-helper cells which in their majority belong to the TH2 type (Romagnani, Immunol Today 13 (1992) 379-381). Therapy of Type I allergic diseases is currently performed by pharmacological treatment and by specific immunotherapy. Specific immunotherapy has been established already early in this century (Noon, Lancet 1 (1911) 1572-1573) and involves the systemic application of increasing doses of allergens for extended periods. Although specific immunotherapy is recognized as effective treatment, the occurrence of anaphylactic side effects represents one of the major disadvantages of this therapy. To reduce anaphylactic reactions the use of T-cell epitopes has recently been proposed for allergen specific immunotherapy (Briner et al., Proc. Natl. Acad. Sci. USA 90 (1993) 7608-7612, and Norman, Curr. Opin. Immunol 5 (1993) 986-973). Allergens harbour a great variety of different T-cell epitopes (Ebner et al., J. Immunol 150 (1993) 1047-1054; Joost-van-Neerven et al., J. Immunol. 151 (1993) 2326-2335; and Schenket al., J. Allergy Clin. Immunol. 96 (1995) 986-996) which may overlap with continuous IgE-epitopes. To prevent crosslinking of effector cell (mast cell, basophil) bound IgE and mediator release, T-cell epitopes and IgE epitopes need to be dissected. Following the concept of converting a major allergen into a T-cell vaccine we have selected Bet v 1 (Breiteneder et al., EMBO J. 8 (1989) 1935-1938), the major birch pollen allergen as a model. Bet v1 was selected because epitope analysis indicated that it forms conformational IgE epitopes (Visco et al., J. Immunol. 157 (1996) 956-962; and Laffer et al., J. Immunol. 157 (1996) 4953-4962). In addition Bet v1 represents one of the most common allergens which is recognized by 95% of tree pollen and food allergic individuals and almost 60% of them are sensitisized exclusively against Bet v1 (Jarolim et al., Allergy 44 (1989) 385-394). The cDNA coding for Bet v1 has

recently been isolated (Breitenederet al., EMBO J. 8 (1989) 1935-1938) and recombinant Bet v1 was expressed in Escherichia coli (Valenta et al., J. Allergy Clin. Immunol. 88 (1991) 889-894; and Ferreira et al., J. Biol. Chem. 268 (1993) 19574-19580). Recombinant Bet v1 has been shown to possess similar IgE-binding capacity as natural Bet v1 and shares IgE as well as T-cell epitopes with Bet v1 homologous proteins present in pollen from various trees and plant derived foods (Ebner et al., J. Allergy Clin Immunol. 95 (1995) 962-969; Ebner et al., J. Immunol 150 (1993) 1047-1054; and Schenk et al., Eur. J. Biochem. 224 (1994) 717-724). The biological activity of the recombinant Bet v1 has been demonstrated by histamine release experiments and by skin prick testing of allergic patients (Valenta et al., J. Allergy Clin. Immunol. 91 (1993) 88-97; Pauli et al., J. Allergy Clin. Immunol. 98 (1996) 1100-1109; and Menz et al., Clin. Exp. Allergy 26 (1995) 50-60).

The invention.

The first aspect of the invention is an immunogen derived from a protein allergen. It has a strongly reduced anaphylactic ability compared to the protein allergen from which it derives and will therefore in the context of the present invention be called non-anaphylactic. The immunogen is characterized in that it comprises:

a. a non-anaphylactic immunogenic recombinant fragment of the protein allergen, said fragment containing an IgG epitope partly but not wholly overlapping an IgE epitope of the protein allergen, said IgE epitope having been broken up by fragment formation;

b. a polymeric form of said fragment, in which form the fragment constitutes the monomeric units;

c. a recombinant polymeric form of said protein allergen in which the protein allergen constitutes the monomeric units.

By the term "a broken up IgE epitope" is meant that the fragment formation has resulted in a fragment that only contains a part of the correponding IgE epitope present in the starting protein allergen. The epitopes in question may be either conformational or linear, with particular emphasis for the IgE epitope being conformational in case of a fragment according to items (a) and (b). Compare Bet v 1 fragments aa 1-74 and 75-160 as described in the experimental part and by Vratala t al., J. Clin. Invest. 99(7) April 1997) 1673-1681.

By polymeric forms means that the immunogen typically comprises 2-10 of the monomeric units defined in (b) and (c). At the priority date results had been obtained with polymeric forms containing 2, 3 and 4 monomeric units.

The various forms a-c may be produced by recombinant techniques to directly give a fragment according to (a), or a polymeric form according to (b) or (c). For (b) the polymeric form may also be accomplished by covalently linking two or more identical recombinant fragment molecules to a common carrier molecule. In the final immunogen that is to be used for hyposensitization therapy or in vitro assays, the fragment according to (a) and the polymeric forms according to (b) and (c) may have been linked to a carrier in order to increase the immunogenicity. In case this carrier is a protein and one wants to have a linear immunogen it is possible to produce the immunogen in one step by expression of the corresponding gene construct in the appropriate host cell, such as aa procaryotic (e.g. E. coli) or eucaryotic (yeast or a mammalian cell line) cell. See further Scheiner O and Kraft D, Allergy 50 (1995) 384-391; and Valenta R and Kraft D, Current Opinion in Immunology 7 (1995) 751-756.

By the use of recombinant techniques it is easy to introduce oligopeptide linkers between each monomeric unit of the

polymeric form of the immunogen according to items (b) and (c). Suitable amino acid residues in the linker may be selected among hydrophobic or hydrophilic or among basic, acid or neutral amino acids. Hydrophobic amino acids are trp, gly, ala, phe, pro, met, val, leu,and ile. Hydrophilic amino acids are for instance gln, ser, gly, glu, pro, his and arg. The length of the oligopeptide linker typically is an integer in the interval 0-30, such as in the interval 0-10, amino acid residues. At the priority date the preferred linker was the tripeptide leu-val-pro.

In the experimental part the invention is illustrated with the birch pollen allergen Bet v 1.

The second aspect of the invention is specific hyposensitization therapy. This therapy may be performed as known in the art for protein allergens and encompasses administering repeatedly to the mammal, typically a human individual, suffering from type I allergy against the protein allergen an immunogen that is capable of raising an IgG immune response against the protein allergen. Administration may be done systemically, for instance by injection, infusion, i etc, but also the oral route has been suggested in order to expose the intestinal part of the immune system. The immunogen may be admixed with suitable adjuvants such as aluminium oxide. Se further Norman PS, "Current status of immunotherapy for allergies and anaphylactic reactions" Adv. Internal. Medicine 41 (1996) 681-713.

A third aspect of the invention is to use the immunogen of the first aspect, in particular according to item (c) as an antigen in an immunoassay for detecting specific antibodies of the IgA, IgD, IgE, IgG or IgM class directed against the protein allergen or protein allergens from which the immunogen derives. Appropriate assays variants involve formation of a ternary immune complex between the immunogen,

sample antibody and an antibody directed against the Ig-class of interest. The sample may be any Ig-containing biological fluids, for instance a blood derived sample (serum, plasma, whole blood), CSF, etc.

The invention will be defined in the attached claims that are part of the specification. The invention will now be illustrated by two non-limiting patent examples.

EXPERIMENTAL PART

Example 1. Bet v 1 polymers

Construction of the Bet v 1-polymers.

The Bet v 1-cDNA (Breiteneder et al., "The gene coding for the major birch pollen allergen Bet v 1 is highly homologous to a pea resistance response gene", EMBO J. 8 (1989) 1935-1938) was PCR-amplified with the following oligonucleotide primers:

Bet v 1-dimer:

For construction of the first Bet v 1-segment:

5'GAG GAA TTC *CAT ATG* GGT GTT TTC AAT TAC3'
Eco R I Nde I

5'CGG GGT ACC AAG TTG TAG GCA TCG GAG TG3'
Kpn I

For construction of the second Bet v 1-segment:

5'CGG GGT ACC GAT GGG TGT TTT CAA TTA C3'
Kpn I

5'CCG GAA TTC CCG *CTC GAG* CTA TTA GTT GTA GGC ATC GGA GTG3'
Eco R I Xho I

Bet v 1-trimer:

The first Bet v 1-segment: The same primers were used as for construction of the first segment of Bet v 1-dimer.

Second Bet v 1-segment:

Sequence Id No 1:

5'CGG GGT ACC GAT GGG TGT TTT CAA TTA C3'

Kpn I

Sequence Id No 2:

5'CGG AAT TCA *CTA GTG* GGT TGT AGG CAT CGG AGT G3'

Eco R I Spe I

Third Bet v 1-segment:

Sequence Id No 3:

5'CCG GAA TTC GGA *CTA GTA* ATG GGT GTT TTC AAT TAC3'

Eco R I Spe I

Sequence Id No 4: 5'CGG AAT TCG TTG TAG GCA TCG GAG TG3'

Eco R I

Protocol for PCR-amplification: Reaction mix (GeneAmp PCR kit, Perkin Elmer, Branchburg, N.J. USA): 44µl H_2O dd, 10xl 10x PCR buffer, 4µl 5mM dATP, 4µl 5mM dCTP, 4µl 5mM dGTP, 4µ 1 5mM dGTP, 4µl 25mM $MgCl_2$, 3µl 10xM primer 1, 3µl 10xM primer 2, 10µl 1ng/µl Bet v 1. 10x PCR-buffer: 100mM Tris-HCl, pH8.3, and 500 mM KCl. The reaction mixture was heated for 5 minutes at 94°C, afterwards 35 cycles of 1min at 94°C, 2min at 40°C, and 3 min at 72°C were performed. During the first cycle 10µl of AmpliTaq DNA Polymerase (2.5 U/10µl) were added.

After PCR-amplification, the PCR-products were digested with the corresponding restriction enzymes. Primers which contained addditional Eco R I sites, were digested first with Econ R I to facilitate subcloning. Digested fragments were purified using Nick columns (Pharmacia Biotech Ab, Uppsala, Sweden), and ligated into pET-17b plasmids (Novagen, Madison, USA). The plasmid, containing the first Bet v 1-segment, was further digested with Kpn I/Xho I in

the case of Bet v 1-dimer, or with Kpn I/Spe I in the case of Bet v 1-trimer, to obtain vectors, in which the second Bet v 1-segments could be incorporated. In the case of Bet v 1-trimer, this construct was further digested with Spe I/Eco R I and the third Bet v 1-segment was added.

Expression and purification of recombinant Bet v 1-polymers.

Recombinant Bet v 1-dimer and recombinant Bet v 1-trimer were expressewd in E. coli BL21 (DE3) by induction with 0.5 mM isopropyl beta-thiogalactopyranoside at an OD600 of 0.5-0.8 in liquid culture (LB-medium) for 5h at 37°C. E. coli cells were the harvested by centrfugation and washed to remove the culture medium.

LB-medium: 10g sodium chloride, 10g peptone, 5g yeast extract, pH 7.5 with NaOH,autoclaved prior to use.

Purification. Recombinant Bet v 1-polymers were expressed as inclusion bodies and isolated as described (Vrtala et al., "Immunologic characterization of purified recombinant timothy grass pollen (Phleum pratense) allergens (Phl p 1, Phl p 2, Phl p 5)", J. Allergy Clin. Immunol. 97n (1996) 781-786. Inclusion bodies were solubilized with 8M urea, 10mM Tris, pH 8, 1mM EDTA, 5mM beta-mercaptoethanol, diluted with 10mM Tris, pH 8, to a concentration of 6M urea and centrifuged for 15min at 10,000g to remove insoluble material. The supernatant, containing the recombinant protein, was dialyzed to a final concentration of 2M urea. After centrifugation (15min, 10,000g), the supernatant was applied to a column packed with DEAE Sepharose (Pharmacia Biotech AB, Uppsala, Sweden), and the protein was eluted with a 0-0.5M NaCl-gradient. Fractions, containing the recombinant protein which was > 80% pure, were dialyzed against 6M nurea, 10mM NaH_2PO_4, pH 4.8, and rechromatographed on a column packed with SP Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) Fractions, containing recombinant Bet v 1-dimer or recombinant Bet v 1-

trimer of > 95% purity were dialyzed against 10mM Tris, pH 7.5 and stored at -20°C until used.

Results of studies on Bet v 1 polymers

Figure 1. Construction of the Bet v 1 polymers.
The Bet v 1-cDNA (Breiteneder et al., EMBO J. 8 (1989) 1935-1938) was PCR-amplified with oligonucleotide primers containing different restriction enzyme cleavage sites. The PCR-products were then ligated as indicated in the figure and subcloned into the plasmid pET-17b (Novagen, Madison, USA).

Figure 2. Coomassie stained SDS-PAGE gel showing purified recombinant Bet v 1-monomer and Bet v 1-polymers.
Lane M: Molecular weight marker; lane 1 contains 3µg purified, recombinant Bet v 1 monomer, lane 2 3µg purified, recombinant Bet v 1-dimer, lane 3 3µg purified recombinant Bet v 1-trimer and lane 4 3µg purified, recombinant Bet v 1-tetramer.
Result: The purified proteins were more than 95% pure. The dissolved proteins were separated from insoluble material by high speed centrifugation prior to loading the samples.

Figure 3. IgE-reactivity of birch-pollen allergic patients with nitro-cellulose-blotted purified recombinant Bet v 1-monomer, dimer and trimer.
Purified recombinant Bet v 1-monomer, dimer and trimer were separated by SDS-PAGE and blotted onto nitro-cellulose. Sera from 8 different birch pollen allergic patients (lanes 1-8) and serum from a non-allergic person (lane 9) were used to detect the blotted allergens. Bound IgE was detected with 125I labelled anti-human >IgE antibodies (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) and visualised by autoradiography.
Result: The IgE-binding capacity of nitrocellulose-blotted Bet v 1-polymers was comparable to Bet v 1-monomer.

Figure 4: Determination of IgE-reactivity of sera from birch pollen allergic patients with Bet v 1-monomer and polymers by ELISA.

Sera from 4 birch-pollen allergic patients (A-D) were diluted 1:2 (1), 1:10 (2), 1:20 (3), 1:40 (4) and 1:80 (5) and tested for IgE-reactivity with purified, recombinant Bet v 1-monomer, Bet v 1-dimer and Bet v 1-trimer. The OD-values are displayed on the y-axis.

Result: Serum IgE from allergic patients bound to Bet v 1-polymers in a comparable manner as to Bet v 1-monomer.

Figur 5. Inhibition of IgE-binding to recombinant Bet v 1-monomer using Bet v 1-polymers.

Sera from 4 birch-pollen allergic patients (A-D) were preincubated with different concentrations (5µg, 500ng, 50ng and 5ng) of purified, recombinant Bet v 1-monomer, Bet v 1-dimer and Bet v 1-trimer. The preincubated sera were then tested for IgE-reactivity to purified, recombinant Bet v 1-monomer by ELISA. The optical densities are displayed on the y-axis.

Result: IgE-binding to Bet v 1-monomer is inhibited by increasing concentrations of the Bet v 1-polymers in a dose dependent manner. The amounts of Bet v 1-polymers needed for inhibition at certain concentrations (50 ng versus 5 ng) was however approximately tenfold higher compared to the monomer.

Figure 6. Serum IgG_1-reactivity of Bet v 1-polymer immunized mice with recombinant Bet v 1.

8 Balb/c mice were immunized monthly with 5µg purified, recombinant Bet v 1-dimer and $Al(OH)_3$ as adjuvant, 8 Balb/c mice were immunized monthly with 5µg purified, recombinant Bet v 1-trimer-$Al(OH)_3$ and blood samples were taken after each immunization. Serum samples obtained after weeks 19 and 25 of immunization and serum taken before immunization (preimmune serum 0 =) were diluted 1:1000 and tested for IgG_1-reactivity with purified, recombinant Bet v 1-monomer

in an ELISA. The symbols represent the OD-values that corresponds to the IgG_1-binding of the 8 different Bet v 1-dimer or Bet v 1-trimer mice.

Result: The Bet v 1-polymers are able to induce high levels of IgG_1-antibodies, which crossreact with Bet v 1-monomer.

Figure 7. Capacity of recombinant Bet v 1-polymers to induce histamine release.

Granulocytes from two different birch pollen allergic patients (a,B9 were incubated with increasing concentrations (0.01 µg/ml, 0.1µg/ml, 1µg/ml and 10µg/ml) of purified, recombinant Bet v 1-monomer, Bet v 1-dimer, Bet v 1-trimer, Bet v 1-tetramer and anti-IgE antibodies as positive control. Histamine release in the cell free supernatant was measured by RIA (Immunotech, Marseille) and is expressed as percentage of total histamine release.

Result: Bet v 1-dimer induced an approximately 2 fold reduced histamine release from patients' basophils compared to Bet v 1-monomer, whereas Bet v 1-trimer and tetramer had an approximately 100 fold reduced capacity to induce histamine release. In the donors tested, Bet v 1-monomer induced maximal histamine release at a concentration of 0.01 µg/ml, Bet v 1-trimer and tetramer at a concentration of 1µ g/ml.

Table 1. Proliferation of Bet v 1 specific T-cell clones with recombinant Bet v 1-polymers.

The full table is given at the end of the descriptive part. T-cell clones from different pollen allergic donors (column 2 shows the initials of the donors) with specificity for different Bet v 1 epitopes (in column 1 the position of the epitopes are indicated) were incubated with purified, recombinant Bet v 1-monomer (column 4), Bet v 1-dimer (column 5), Bet v 1-trimer (column 6) and Bet v 1-tetramer (column 7). As negative control, clones were tested with medium alone (column 3). Proliferation was determined by ^3H

Thymidine uptake and is displayed as counts per minute (cpm) (columns 3-7).

Result: Bet v 1-polymers and Bet v 1-monomer induced comparable proliferation of specific T cell clones.

Table 2. Skin testing with recombinant Bet v 1-monomer and polymers.

The full table is given at the end of the descriptive part. 6 birch-pollen allergic individuals and 4 non-allergic control individuals were skin prick tested on their forearms with natural birch pollen extract, histamine as positive control and with 10µg/ml and 100µg/ml of purified, recombinant Bet v 1-monomer, Bet v 1-dimer and Bet v 1-trimer. The mean wheal diameters (DM) are displayed in the table.

Result: Bet v 1-dimer induced an approximately 10-fold reduced skin reaction in allergic patients compared to Bet v 1-monomer, whereas Bet v 1-trimer induced in some patients no wheal reactions at all, up to a concentration of 100µg/ml. The wheal reaction increased dose dependently with the protein concentrations. The non-allergic control individuals displayed only skin reactions with histamine but not with the Bet v 1-preparations. Both the histamine release assays and the skin tests indicate, that the Bet v 1-polymers have a greatly (up to 100 fold) reduced anaphylactic activity compared to Bet v 1-monomer. The reduction of anaphylactic potential is proportional to the degree of polymerization.

Summary - studies on Bet v 1 polymers.

We expressed in pET 17b plasmids (Novagen, Madison, USA) Bet v 1 as dimer, trimer and tetramer. The Bet v 1-polymers were expressed at high levels in E. coli BL21 (DE3) (Novagen, Madison, USA) and purified to homogeneity. The Bet v 1-polymers retained their IgE-binding capacity, as was shown by immunoblotting and by ELISA. T-cell clones from birch allergic donors, with specificity for Bet v 1 proliferated upon incubation with all the polymers, indicating that the

polymers contain the relevant T-cell epitopes of Bet v 1. Bet v 1-trimer and tetramer had an approximately 100 fold reduced capacity to induce histamine release from patients' basophils and a greatly reduced anaphylactic potential as evaluated by skin testing. Because of the reduction of their anaphylactic activity the Bet v 1-polymers may be considered as safe tools for specific immunotherapy of tree pollen and associated food allergy. allergic patients may be treated with high doses of these derivatives with reduced risk of anaphylactic side effects. The difference of the recombinant polymers to non-anaphylactic T-cell epitope containing allergen derivative is that they contain the IgE-binding sites but have a reduced anaphylactic potential.

Example 2. Mapping the binding site of antibodies in Bet v 1.

Figure 8: Two monoclonal anti-Bet v 1-antibodies (moAb A and B) were used together with three synthetic Bet v 1-derived peptides were used in ELISA. The sequences of th three peptides are shown in the lower part of the figure and corresponds to aa 49-60 (p17), aa 52-63 (p18) and aa 55-66 (p19) of Bet v 1. The peptides were tested for binding to the two Bet v 1 specific monoclonals. The OD values are displaed on the y-axis. Both moAbs bind to the peptides p18 and p19, which are mapped to the first half of Bet v 1.

Table 3. The full table is given at the end of the descriptive part. Monoclonal anti-Bet v 1 antibodies (A,B) inhibit binding of human IgE to recombinant Bet v 1. Dot-blotted Bet v 1 was preincubated with MoAb A and B prior to probing with serum IgE from 60 Bet v 1 allergic individuals. Bound IgE was detected with 125I-labelled anti-human IgE antibodies and quantified by gamma-counting. Inhibition of IgE binding was determined as follows:

$$100-(cpm_1/cpm_2)100 = \%inhibition$$

cpm_1 = count per minutes for incubation with moAb
cpm_2 = count per minutes for incubation buffer
The % inhibition of IgE-binding compared to preincubation with buffer is displayed in the table.

EXAMPLE 3. TWO NON-ANAPHYLACTIC RECOMBINANT FRAGMENTS BET V 1.
See further Vrtala et al., "Conversion of the major birch pollen allergen, Bet v 1, into two non-anaphylactic T cell epitope containing fragments", J. Clin. Invest. 99(7) April 1997) 1673-1681.

METHODS

Sera from allergic patients, antibodies, protein extracts and E. coli strains. Sera from birch pollen allergic patients and control individuals were characterized by RAST and testing with recombinant allergens as described (Valenta et al., J. Allergy Clin. Immunol. 88 (1991) 889-894; Valenta etal., Int. Arch. Allergy Immunol. 97 (1992) 287-294). In addition all patients were characterized by case history and skin pricl test. The mouse monoclonal antibody moab 14 with specificity for aa 40-65 of Bet v 1 is described (Lebecque et al., J. Allergy Clin. Immunol. in press). Natural birch pollen extract was prepared as described (Vrtala et al., Int. Arch. Allergy Immunol. 102 (1993) 160-169). Plasmid pET-17b containing the ampicillin resistance ansd a T7 promotor was obtained from Novagen, Madison, USA. Recombinant Bet v1 fragments were expressed in λDE3 lysogens of E. coli strain BL21 (F^- $ompT r_b$-m_B-) (Studier et al., Meth. Enzymol. 185 (1990) 60-89).

Expression of Bet v 1 (aa 1-74, aa 75-160) fragments in E. coli. Recombinant Bet v 1 fragments (aa 1-74, aa 75-160) were generated to maintain the epitopes (aa 40-65) of murine monoclonal antibodies which inhibited binding of allergic patients IgE to Bet v 1 (Lebecque et al., J. Allergy Clin. Immunol. in press) and in order to preserve major T-cell epitopes which had been mapped using ocverlapping peptides

synthesized according to the Bet v 1 sequence (Ebner et al., J. Immunol. 150 (1993) 1047-1054). The cDNAs coding for fragment aa 1-74 and aa 75-160 were obtained by PCR amplification of the Bet v 1 cDNA using the following oligonucleotide primers (Pharmacia Biotech AB, Upsala Sweden):

Bet v 1 (aa 1-74):

Sequence Id No 5:

5'GGG AAT TCC *ATA TGG* GTG TTT TCA ATT AC3'

Sequence Id No 6:

5'CGG *GGT ACC* TTA CTC ATC AAC TCT GTC CTT3'

Bet v 1 (aa 75-160):

Sequence Id No 7:

5'GGG AAT TCC *ATA TGG* TGG ACC ACA CAA ACT3'

Sequence Id No 8:

5'CGG *GGT ACC* TTA GTT GTA GGC ATC GGA3'

The ECO R I sites which were incorporated in the first primers are underlined, Nde I and Kpn I sites are in italics. To improve subcloning efficiency, PCR-products were first cut with Eco R I and Kpn I, purified by preparative agarose gel electrophoresis, subcloned into Eco R I and Kpn I site of plasmid pEt-17b (Novagen, Madison, USA) and transformed into E. coli BL21 (DE3) (Novagen, Madison, USA) by electroporation. Inserts were then excised with Nde I/Kpn I and subcloned again in plasmid pET-17b and transformed. Colonies expressing the correct fragments were identified by immunoscreening using mab 14 for Bet v 1 aa 1-74 and a rabbit anti-Bet v 1 C-terminal antiserum for Bet v1 aa 75-160. DNA from positive clones was isolated using Qiagen tips (Quiagen, Hilden, Germany) and both DNA strands were sequenced according to Sanger using a T7 polymerase sequencing kit (Pharmacia Biotech AB, Uppsala, Sweden) and ^{35}S dCTP (NEN, Stevehage, UK)(24). Recombinant Bet v 1 (aa 1-74 and Bet v1 (aa 75-160) were expressed in E. coli BL21 (DE3) by induction with 0.5 mM IPTG at an OD600 of 0.5-0.8 in liquid culture for 5 hours at 37°C.

Purification of recombinant Bet v1 (aa 1-74) and Bet v1 (aa 75-160). Bet v1 (aa 1-74) and Bet v1 (aa 75-160) were expressed in inclusion bodies isolated as described (Vrtala et al., J. Allergy Clin. Immunol. 97 (1996) 781-787). Inclusion bodies were solubilized with 8M urea, 10 mM Tris, pH 8, 1 mM EDTA (ethylenediaminetetraacetic acid), 5 mm β-mercaptoethanol, diluted with 10 mM Tris, pH 8 to a concentration of 6 M urea and centrifuged for 15 minutes at 10,000xg to remove insoluble material. The supernatant containing the recombinant protein, was dialyzed to a final concentration of 2M urea. Following centrifugation (15min, 10,000xg), the supernatant was applied to a column packed with DEAE (diethylaminoethyl) Sepharose (Pharmacia Biotech AB) and the protein eluted with a 0-0.5M NaCl concentration gradient. Fractions, containing the recombinant protein which was more than 80% pure, were dialyzed against 6M urea, 10mM NaH_2PO_4, pH 4.8 and rechromatographed on a column packed with SP Sepharose (Pharmacia Biotech AB). Fractions containing recombinant Bet v 1 (aa 1-74) or recombinant Bet v 1 (aa75-160) of greater than 95% purity, were dialyzed against 10mM Tris, pH 7.5 and lyophilized until used.

IgE binding capacity of recombinant Bet v 1 and Bet v 1 fragments. Purified recombinant Bet v 1 and Bet v 1 fragments (aa 1-74, aa 75-160) were tested for IgE-binding capacity by Western blotting and in dot blot assays. For immunoblotting, approximately 1 µg/cm purified protein was separated by SDS-PAGE (Fling et al., Anal. Biochem. 155 (1986) 83-88) and blotted onto nitrocellulsoe according to Towbin (Towbin et al., Proc. Natl. Acad. Sci. USA 76 (1979) 4350-4353). To avoid denaturation of the proteins, dot blot experiments were performed in parallel. One µg of purified recombinant Bet v 1, 1µg of each Bet v 1 fragment and 1µg of bovine serum albumin and human serum albumin (HSA) (negative controls) were dotted on nitrocellulose strips.

Nitrocellulose strips containing Western blotted allergens or the dot blotted proteins were incubated with serum IgE

from allergic individuals, non-allergic control individuals and buffer without addition of serum as described (Valenta et al., J. Exp. Med. 175 (1992) 377-385). Bound IgE antibodies were detected with ^{125}I labelled anti-human IgE antibodies and visualized by autoradiography.

Results: Sera of birch pollen allergic patients reacted with recombinant Bet v 1 but not with Bet v 1 fragments. Sera of grass pollen allergic individuals reacted neither with recombinant Bet v 1 nor with the recombinant Bet v 1 fragments.

Circular dichroism showed that the two Bet v 1 fragments showed no tendency to fold, even in the presence of each other.

Histamine release experiments. Granulocytes were isolated from heparinized blood of birch pollen allergic individuals by dextran sedimnetation (Valent et al., Proc. Natl. Acad. Sci. USA 86 (1989) 5542-5547). Cells were incubated with different concentrations (0.001µg/ml-10µg/ml) of purified rcombinant Bet v 1, recombinant Bet v 1 fragments (aa 1-74, aa 75-160) separately and in equimolar mixture, or anti-human IgE antibodies. Histamine released in the supernatant was measured by radioimuoassay (RIA) (Immunotech, Marseille, France) (Valenta et al., J. Allergy Clin. Immunol. 91 (1993) 88-97). Total histamine was determined in cell lysates after freeze thawing. Results were obtained as mean values from triplicate determinations and expressed as percentage of total histamine release.

Results: Recombinant Bet v 1 fragments have approximately 1000 fold reduced capacity to induce histamine reslease from patients basophils compared to recombinant Bet v 1. An equimolar mixture of both Bet v 1 fragments did not induce significant release of histamine compared to each of the tested fragments.

Skin testing. Skin prick tests were performed on the individuals' forearms by placing µl of each solution (Pauli et al., J. Allergy Clin. Immunol. 97 (1996) 1100-1109; Menz et al., Clin. Exp. Allergy 26 (1996) 50-60). Recombinant Bet v 1 and recombinant Bet v 1 fragments were freshly dissolved in a 0.9% w/v sterile sodium chloride solution at concentrations of 100µg/ml and 10µg/ml. As controls birch pollen SQ (standard quality) extract, sodium chloride solution (negative control) and histamine hydrochloride (positive control)(ALK, Horsholm, Denmark) were used. Each drop was pricked with a fresh prick lancette (ALK, Horsholm, Denmark) and results were recorded after 20 minutes with a ball point pen by transferring the wheal area with a tape paper and by photography. The mean wheal diameter (Dm) was calculated by measuring the maximal longitudal diameter (D9 and the maximal transversal diiameter (d) according to the formula (D+d)/2=Dm.

Results: The two recombinant Bet v 1 fragments, neither alone nor in combination, do not elicit anaphylactic skin reactions compared to the intact recombinant Bet v 1.

Table 1: Proliferation of Bet v 1 specific T-cell clones with recombinant Bet v 1-polymers.

1 Epitop Bet v 1	2 TCC	3 Control	4 Bet v 1	5 Bet v 1-dimer	6 Bet v 1-trimer	7 Bet v 1-tetramer
1-15	CGE 147	15567	47570	97939	67299	79741
1-18	HC 26/II	1264	9977	32667	14170	22178
10-27	WF 110/III	87	6402	12571	5823	9542
10-27	WF 110/III	146	3575	13340	5428	6961
10-27	WF 121/III	287	3914	22099	5117	13000
11-27	TF 7B	359	10492	42352	9869	29900
35-48	HC 3/III	40.7	10499	21301	15761	25609
64-75	CGE 110	612	107103	121178	96135	117930
64-75	CGE 31	2937	71176	55728	38955	67625
64-75	CGE 33	3096	99633	85438	80077	91755
77-93	WF 29R	143	12638	28579	14576	14677
77-93	GZ 17M	172	61463	90586	54988	84237
88-10	CGE 34	515	16045	20531	14176	15217
93-110	TF 1M	438	21423	29741	11500	23454
106-120	WF 9/III	305	43203	81605	32735	65592
109-120	WD 7/III	130	53362	41875	50489	48601
110-128	HC 33/II	134	18099	46022	17917	42051
112-123	WF 112/III	85	10494	12778	7585	11106
112-123	WF 97/III	91	4569	6884	3352	5950
127-138	GZ 10A	182	3347	8379	3227	6645
141-156	TF 10A	215	4862	4438	2232	57
141-156	RR4R	1416	88361	85594	102303	117122
141-156	SAZ 10/IV	612	5121	3830	5207	3979

Table 2: Skin testing with recombinant Bet v 1-monomer and polymers

Individual	Histamine	birch	Bet v 1 monomer 10µg/ml	Bet v 1 monomer 100µg/ml	Bet v 1 dimer 10µg/ml	Bet v 1 dimer 100µg/ml	Bet v 1 trimer 10µg/ml	Bet v 1 trimer 100µg/ml
birch pollen allergic patients								
MS	8	5.5	4	7	3	6	0	0
SF	6	7	8	12	7.5	8	2	5.5
PSt	8	7	6.5	16	6	7	2	4.5
SO	6.5	5.5	5.5	14	0	4.5	0	3.5
SS	4.5	8	5.5	9	0	4	0	0
MD	5.5	9.5	7	11.5	4.5	7	0	5
non-allergic controls								
TB	6	0	0	0	0	0	0	0
VR	8.5	0	0	0	0	0	0	0
CD	6.5	0	0	0	0	0	0	0
TL	9	0	0	0	0	0	0	0

patient #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Inhibition of IgE binding in %	moAb A	49	-	-	57	93	96	-	41	-	41	27	-	29	47	-
	moAb B	96	-	-	45	-	97	-	31	-	45	24	-	-	26	-

patient #		16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
inhibition of IgE binding in %	moAb A	19	21	35	-	36	-	-	10	20	51	30	-	30	-	55
	moAb B	24	25	12	14	21	-	-	-	22	31	33	-	24	-	50

patient #		31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Inhibition of IgE binding in %	moAb A	10	28	8	18	23	23	3	-	46	22	-	8	30	80	33
	moAb B	4	90	5	59	87	97	13	-	18	19	65	80	10	94	17

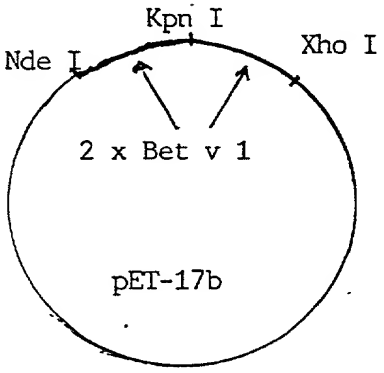
patient #		46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Inhibition of IgE binding in %	moAb A	-	6	54	30	36	12	-	-	-	72	31	1	12	38	-
	moAb B	-	31	97	-	35	8	-	-	-	67	41	-	10	28	-

CONSTRUCTION OF THE BET V 1 POLYMERS

Bet v 1-Dimer

Sequence Id Nos 9 and 10, respectively:

```
ATG.......AAC TTG GTA CCG ATG.....AAC TAA
Met        Asn Leu Val Pro Met      Asn End
   Bet v 1                    Bet V 1
```



Bet v 1-Trimer

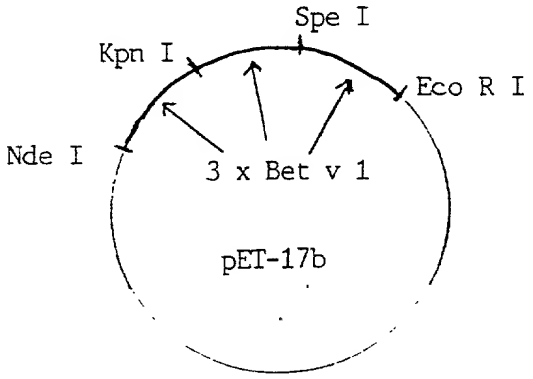
Sequence Id Nos 11 and 12, respectively:

```
ATG.....AAC TTG GTA CCG ATG.....AAC CCA CTA GTA ATG.....AAC
Met.....Asn Leu Val Pro Met.....Asn Pro Leu Val Met.....Asn
  Bet v 1                  Bet v 1                  Bet v 1

GGA TTC TGC AGA TAT CCA TCA CAC TGG CGG CCG CTC GAG CAG ATC
Glu Phe Cys Arg Tyr Pro Ser His Trp Arg Pro Leu Glu Gln Ile

CGG CTG CTA ACA AAG CCC GAA AGG AGG CTG AGT TGG CTG CTG CCA
Arg Leu Leu Thr Lys Pro Glu Arg Lys Leu Ser Trp Leu Leu Pro

CCG CTG AGC AAT AAC TAG
Pro Leu Ser Asn Asn End
```



Bet v 1-tetramer

Sequence Id Nos 13 and 14, respectively:

```
ATG.....AAC TTG GTA CCG ATG.....AAC CCA CTA GTA ATG.....AAC
Met.....Asn Leu Val Pro Met.....Asn Pro Leu Val Met.....Asn
  Bet v 1                 Bet V 1                 Bet v 1

GAA TTC ATG.....AAC TAA
Glu Phe Met.....Asn End
          Bet v 1
```